Journal of Medical Virology 51:242–251 (1997)

Avidity Determination of IgG Directed Against Tick-Borne Encephalitis Virus Improves Detection of Current Infections

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Recently, avidity determination of IgG has been introduced successfully into virus serology as an additional and specific means for confirmation or exclusion of current infections. This simple and highly reproducible method can compensate for problems arising by classical serology, which include lack of detectable IgM responses during primary infections and persistent IgM responses after past infections. We show that avidity determination can be applied successfully for serological diagnosis of TBEV infection. Using the urea denaturation method, primary TBEV infections showed anti-TBEV IgG of low avidity (avidity index < 0.4), whereas sera from individuals with past infections exhibited high avidity IgG. The retrospective analysis of cases with clinical symptoms of TBEV infection in the absence of detectable anti-TBEV IgM showed that a significant number of these cases (5/45) had anti-TBEV IgG of low avidity, indicating current infection. We recommend the use of avidity determination as a method for routine TBEV serology. ***J. Med. Virol. 51:242–251, 1997.***

KEY WORDS: IgG; avidity determination; TBEV infection

INTRODUCTION

Determination of IgM directed specifically against viral antigens is regarded as the classical method of recognition of current viral infections. This approach is valid as long as the IgM response follows the typical pattern, i.e., if IgM appears before or together with the specific IgG response and decreases below the level of detection later. However, virus serology based solely on determination of IgM leads not infrequently to false conclusions, since the IgM response may exhibit an irregular pattern [Schillinger et al., 1993; Bauer, 1995, 1996]. IgM may be detectable in serum at low titers or for a very short period of time only [De Renzo et al., 1994; Brackmann et al., 1994; Johnson et al., 1994; Brytting et al., 1992]. In a small number of cases, the IgM response in serum may be detectable later than IgG ("delayed IgM response") [Bauer 1996; Nikoskelainen et al., 1974; Zaaijer et al., 1993; Hummel et al., 1992; Schillinger et al., 1993; Ho et al., 1992; Glimaker et al., 1992]. Therefore, detection of IgM can be missed if the serum sample is taken too late or too early and can thus cause false exclusions of current infections. There are also many reports on IgM responses that persisted for months or even years after the primary infection [Thomas et al., 1992; Erdman et al., 1991; Roggendorf et al., 1981; Hofmann et al., 1983 a; Zaaijer et al., 1993; Hedman et al., 1989; Lundkvist et al., 1993]. This situation may result in a false-positive diagnosis of current infection. In order to avoid the bias of IgM serology, avidity determination of IgG has been introduced successfully into virus serology recently as an additional and specific means for confirmation or exclusion of current infections.

Increase of antibody affinity during the maturation of the humoral immune response is a general immunological phenomenon [Eisen and Siskind, 1964; Werblin et al., 1973; Brown et al., 1984; Hedman et al., 1989a,b]. Increasing affinity results in increasing stability of the antigen-antibody complex in the presence of dissociating agents. In recent years the term "avidity" has been commonly used to describe the degree of resistance against dissociation by agents such as urea or diethylamine [Hedman and Seppälä, 1988; Thomas and Morgan-Capner, 1988; Hedmann and Rousseau, 1989b]. Avidity determination can be carried out easily by the treatment of antigen-antibody complexes with high concentrations of urea [Thomas and Morgan-Capner, 1991]. Determination of antibody avidity has been used successfully in many viral systems for distinction between primary infections and reactivations [Blackburn et al., 1991; Hedman and Seppälä, 1988; Hedman and Rousseau, 1989b; Hedman et al., 1991; Kangro et al., 1991; Lehtonen and Meurman, 1982;

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Accepted 21 October 1996

Morgan-Capner and Thomas, 1988; Thomas and Morgan-Capner, 1988; Ward et al., 1993; Andersson et al., 1994; Vetter et al., 1994]. Avidity determination has been especially useful for EBV serology, as it allowed differentiation between primary EBV infections, characterized by negative anti-EBNA-1, and cases of past infection, but with lost anti-EBNA-1 (secondary negative anti-EBNA-1) due to suppression of cellular immunity [Vetter et al., 1994]. This strategy has thus increased the significance of EBV serology [Bauer, 1995].

In endemic areas, tick borne encephalitis virus (TBEV) infection is one of the most frequent causes of viral meningitis and encephalitis. Specific tests for diagnosis or exclusion of current TBEV infection are therefore required for differential diagnosis and for the determination of immunity. IgG and IgM-specific ELISAs have been used successfully in recent years [Heinz et al., 1981; Roggendorf et al., 1981; Frisch-Niggemeyer, 1982; Hofmann and Popow-Kraupp 1982; Hofmann et al., 1983a,b; Grandien et al., 1984; Schmitz and Emmerich, 1984; Hofmannn et al., 1985; Grubhoffer et al., 1988]. Evaluation of TBE virus specific IgM ELISAs pointed to the problem of persistent TBE virus IgM [Roggendorff et al., 1981; Hofmann et al., 1983a], which might lead to false-positive determinations of current viral infection in some cases. The usefulness of avidity determination was investigated as a basis for unequivocal TBEV serology.

MATERIALS AND METHODS

Patients and Control Persons

Sera used in this study were obtained from people living in the area around Freiburg (South West Baden-Württemberg, Germany), an area known to be endemic for tick-born encephalitis virus. All the sera were incubated at 56°C for 30 min to reduce the risk of coincidental HIV infection and had been tested in a commercially available TBEV IgG and μ-capture TBEV IgM ELISA (Immuno Diagnostics, Heidelberg) using the procedures and criteria recommended by the manufacturer. Aliquots of the sera were stored at −20°C and were used for avidity determination.

Group A consisted of 91 sera from 61 patients (mean age 43.3 years) with clinical symptoms of current TBEV infection and positive results for TBEV IgG and TBEV IgM.

Group B consisted of 32 sera from 30 control persons (mean age 28.7 years) with past TBEV infection or vaccination (serum taken half a year after infection or vaccination), with positive TBEV IgG and negative TBEV IgM.

Group C consisted of 49 sera from 45 patients (mean age 24.7 years) with tick bite, clinical symptoms of current TBEV infection (high fever, severe headache, meningitis, peripheral paralytic symptoms), positive TBEV IgG but negative TBEV IgM. Sera had been taken less than 25 days after onset of clinical symptoms.

Urea in PBS for Avidity Determination

The amount of urea calculated for the molarity required (usually 7M) was dissolved in prefixed PBS buffer at the respective volume wanted. The solution was gently warmed (35–40°C) and stirred until all the urea was dissolved. Due to the large volume of the urea required, the final concentration of salts in "PBS plus urea" is somewhat lower than in pure PBS. This difference is without relevance for the assay.

ELISA for the Determination of TBEV IgG Avidity

This study was conducted with a commercially available TBEV IgG ELISA obtained from Immuno Diagnostics (Heidelberg) and carried out according to the manufacturers instructions. The final results were confirmed using the ELISA from another manufacturer (Fresenius, Bad Homburg).

Briefly, 1:100 dilutions of sera (or as indicated in the figure legends) were incubated in the antigen-coated assay system for 60 min at room temperature and then the serum was removed.

Control assays were then washed three times with a wash buffer. Avidity assays were incubated with 7 M urea in PBS for 3 min, followed by two wash steps with wash buffer. The assays were continued under standard conditions (incubation with enzyme-labelled goat-anti-human IgG, three wash steps, incubation with chromogen, stopping by H_2SO_4). OD was determined at 450 nm. The OD of the blank assay (lower than 0.02) was subtracted.

Calculation of the avidity index

A) Shift method. This method requires testing of serially diluted serum, both under standard conditions and with urea treatment after the first incubation. The OD values of both curves are plotted against the dilution. The axis showing the dilutions is arranged in a logarithmic scale. The avidity index correlates inversely to the degree of shifting necessary to match both curves; i.e., if the curve obtained for control and urea treatment are identical, the avidity index is 1. If the urea curve has to be moved 3 dilution steps (of 1:2), the avidity index is 1:8 (0.125), and so on. A fine determination of the avidity index can be made by dividing the dilution of the urea-treated curve necessary for a defined OD (preferably in the range of 0.25–0.6 fold of the maximal OD) by the respective dilution of the control curve at the same OD.

B) Single point determination. If the test system is standardized and performed with suitable precision, single point determinations are sufficient for avidity determination. The OD obtained from the urea-treated assay (blank value subtracted) is divided by the respective OD of the control assay. The OD of the control assay must be located within the linear range of the assay; in our system this was between 0.6 and 1.8.

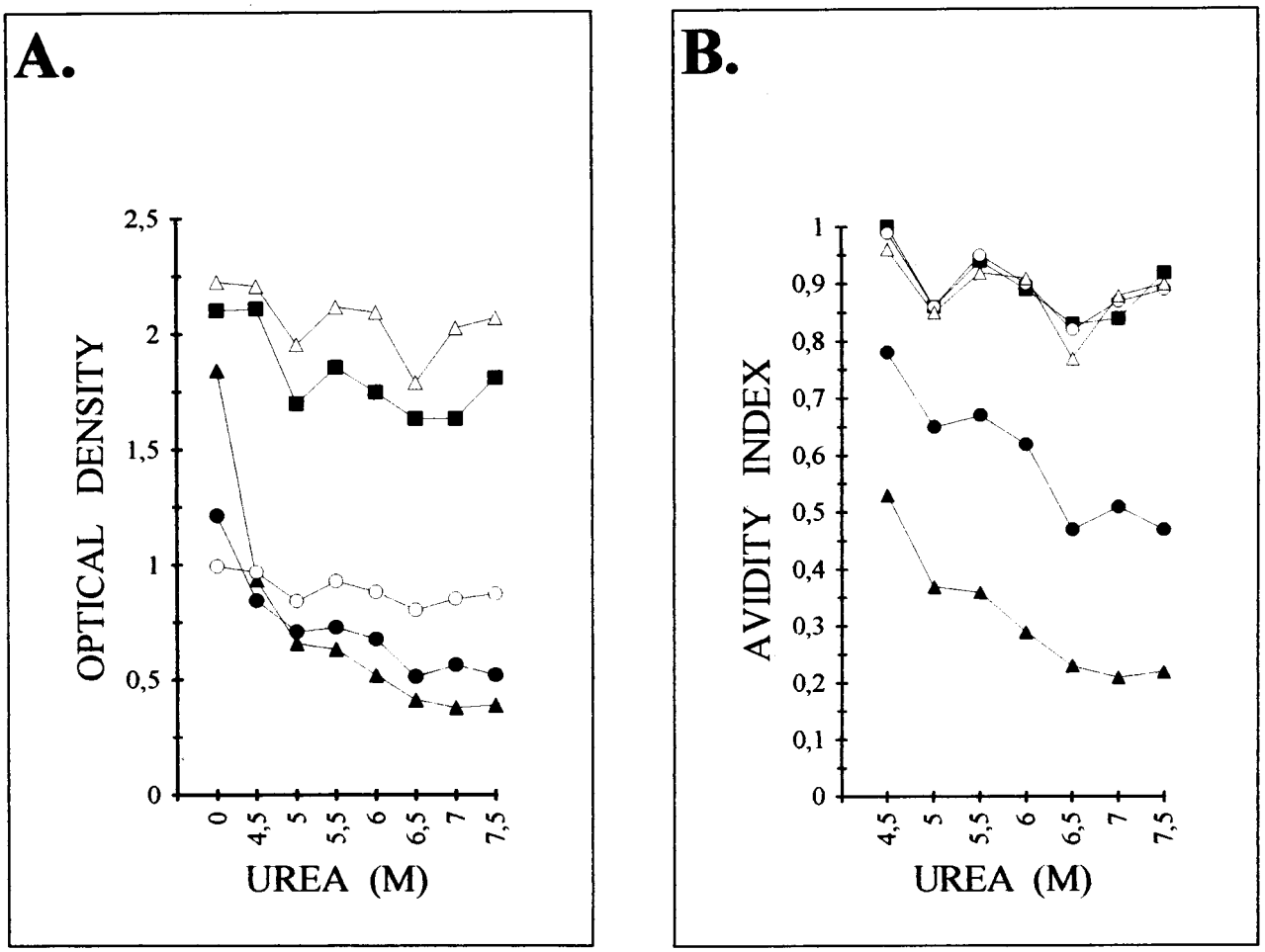


Fig. 1. Determination of the optimal urea concentration for avidity determination of TBEV IgG. Sera from patients with past or current TBEV infection were found to show high, intermediate, or low avidity in a screening test, using one serum dilution and washing with 6M urea for 3 min after the first incubation. These sera were then incubated in parallel tests in a commercial TBEV ELISA for 60 min at a dilution of 1:100. After removal of the serum, parallel assays were either washed with buffer or with the indicated concentration of urea in PBS for 3 min, followed by three wash steps with buffer. The ELISAs were then processed as usual. **A:** The OD values measured are plotted against the urea concentration applied. **B:** The avidity indices calculated from the ratio of ODs obtained with urea treatment compared to control are shown. Closed triangles: low avidity; closed circles: intermediate avidity; open triangles, open squares and closed squares: high avidity.

Alternatively, the OD values obtained for single point determinations, treated with urea and untreated, can be compared to a standard dilution curve and arbitrary units of antibody are calculated. The ratio of the units calculated for the treated assay divided by the units for the control represents the avidity index.

RESULTS

The optimal urea concentration for avidity determination must be determined for each individual test system [Andersson et al., 1994]. Therefore, sera from five patients with current or past TBEV infection which had been recognized in pretests as being of low, intermediate or high avidity were initially tested for avidity of TBEV-specific IgG in a commercially available TBEV ELISA. After the incubation of serum and antigen, parallel assays were either washed with buffer or with buffer containing increasing concentrations of urea. As can be seen in Figure 1, the plateau of removal of antibodies of sera derived from current infection was reached at urea concentrations of 7–7.5 M, whereas the binding of antibodies of sera from past infections was not influenced substantially under these conditions. Therefore, further tests were undertaken using a wash step with 7 M Urea for 3 min.

The classical and most precise way to determine the avidity index is achieved by determination of the shift between the titration curves of the assays treated with urea to the standard titration. As shown in Figure 2 A/B, a serum obtained 15 days after onset of clinical symptoms due to TBEV infection showed an avidity index of 0.06, as determined by the shift assay. One year later, avidity of TBEV IgG had matured, showing an index of approximately 0.7. This approach requires 16 tests per serum and thus raises economical problems if it is used routinely. As an alternative simplification, the OD values of different single dilutions of a serum, assayed without or with urea treatment were

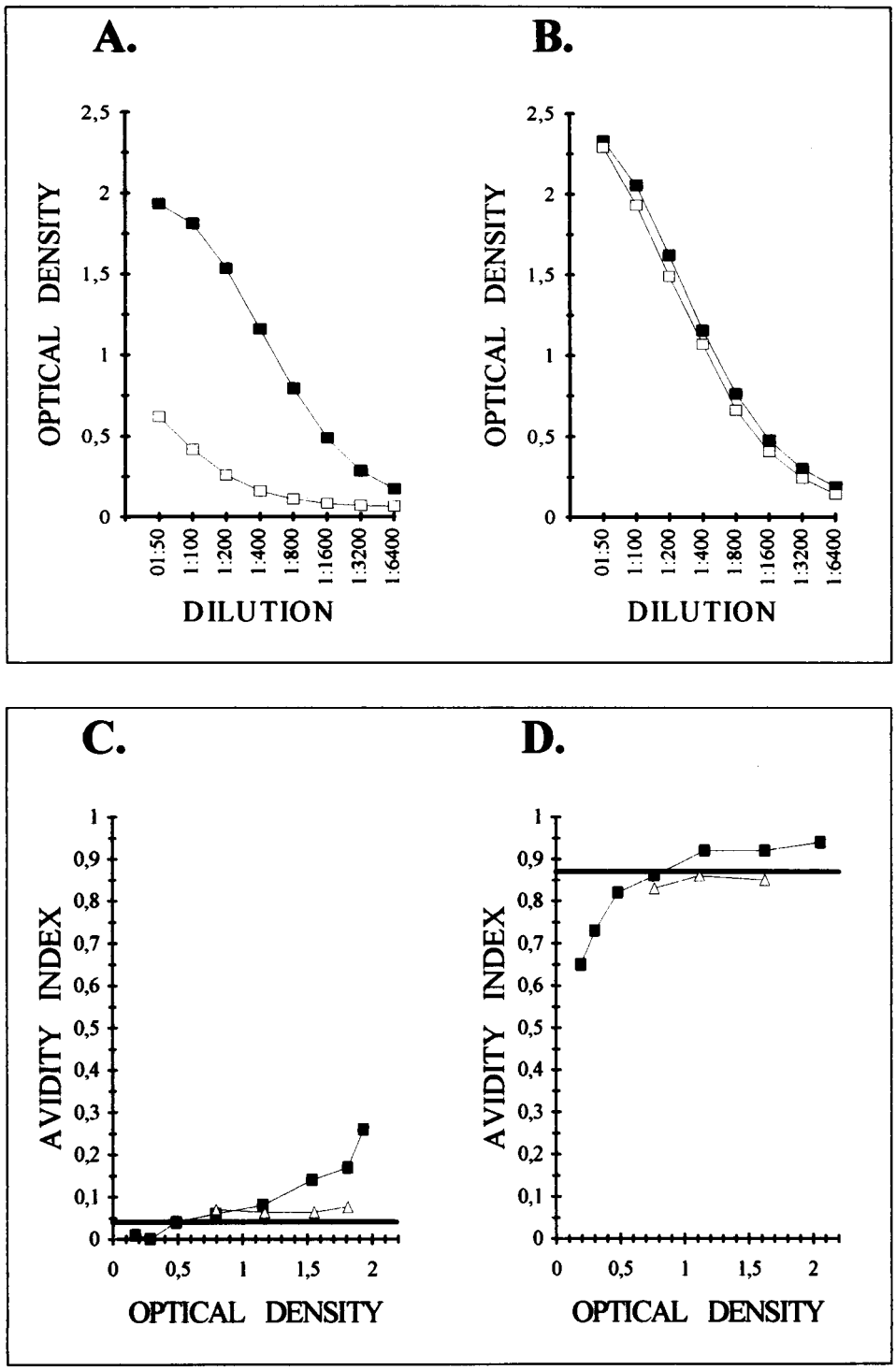


Fig. 2. Determination of avidity indices of TBEV IgG. Sera taken from a patient with clinically overt TBEV infection taken at day 15 or 370 after onset of clinical symptoms were serially diluted and applied to a TBEV IgG ELISA. After the first incubation, parallel assays were either washed with buffer or treated with 7 M urea in PBS, followed by a buffer wash step. Assays were then processed as usual. **A,B:** The OD values obtained are plotted against the respective dilutions. Closed squares: buffer-treated controls; open squares: urea-treated assays. **C,D:** The determination of avidity indices based on the data shown in A and B. Closed squares: the avidity indices were calculated by dividing the corresponding OD values obtained after urea treatment by the control value. The avidity indices are plotted against the OD of the respective control. Open triangles: The avidity indices were determined by calculating the respective concentration of bound antibodies based on individual dilutions in comparison to a standard dilution curve, as described under materials and methods. Bar: The avidity index was calculated by shifting the curve obtained after urea treatment to the control curve.

calculated. As can be seen in Figure 2C/D, this mode of approach leads to results that correspond reasonably to the results obtained by the more laborious shift method. Importantly, calculations have to be restricted to dilutions where OD values obtained without application of urea are between 0.6 and 1.8. At lower or higher OD values the avidity indices calculated are considerably lower or higher than the correct value.

To evaluate the kinetics of maturation of avidity after primary TBEV infection, sera of patients where a sequential series of sera taken at defined intervals after onset of clinical symptoms were available, were tested in the absence and presence of urea treatment. As shown in Figure 3, sera taken early after the onset of clinical symptoms were typically of low avidity. Maturation occurred with a high degree of variability between individual patients. Within one month, avidity may either increase significantly or remain at a low level. In contrast, sera from past infections were uniformly of high avidity.

For an evaluation of usefulness and significance of avidity determination of TBEV IgG, sera from three groups of individuals were tested: a) patients with clinical symptoms of current TBEV infection and both positive TBEV IgG and IgM; b) individuals with past TBEV infection or vaccination; and c) patients with clinical symptoms suspicious of current TBEV infection but lacking detectable TBEV IgM. Based on standard serology, the latter group had been diagnosed previously as "no indication for TBEV infection". As shown in Figures 4 and 5, the vast majority of cases from group A exhibited low avidity IgG, whereas 94% of cases from group B showed high avidity. In both groups, there was a small percentage of borderline cases. Group C differentiated into a majority of cases with high avidity, and a substantial number of cases (n = 5) with low avidity. The percentage of borderline cases was higher in group C than in the two other groups. This result demonstrates that avidity determination can detect IgM-negative cases of current infection, as well as cases borderline with respect to avidity of IgG, which are indicative of recent TBEV infection. Interestingly, the situation seen for TBEV was similar to the position determined recently for primary EBV infections [Vetter et al., 1994] (Fig. 5).

DISCUSSION

Our data demonstrate that available commercial TBEV IgG ELISA kits are suitable for avidity determination. Optimization of the urea wash step showed that treatment of antigen-antibody complexes for 3 min with 7 M urea differentiated well between high avidity antibodies, which were not removed, and low avidity antibodies, which were removed from the antigen.

Titration of sera and subsequent parallel wash procedures with buffer or urea represents the most precise way to determine the avidity index which is indicative of the degree of avidity maturation of IgG. This approach will not be suitable for routine serology, since the costs per serum tested would increase to a level not tolerated by the public health system. It will therefore be restricted to problematic cases with avidity indices close to borderline. We evaluated a simplified method for avidity determination, which is based on the calculation of ratios of OD values obtained in assays treated with urea or buffer. The data show that this method gives an acceptable approximation to the optimal values, provided the OD values of the untreated assay are in the range of 0.6 to 1.8. In this range of OD values, the ratio of individual points of measurement corresponds quite well to the ratio obtained by shifting the respective curves. At lower OD values, the avidity indices calculated by individual assays result in an underestimation of the true avidity index, due to the shape of the OD titration curves. At higher OD values, the avidity indices obtained by the simple procedure represent overestimations, as the OD curve is no longer proportional to the amount of antibody bound. Therefore, determination of avidity by the simplified method may require pretesting of the respective sera to determine the optimal dilution which will lead to OD values in the suitable range for the untreated control. It is important to note that the optimal OD values may change slightly between test kits of different producers and also possibly between batches of ELISA plates from the same producer. Therefore, before routine application, individual batches of ELISA plates have to be tested once with serial dilutions of standard sera of current and past infection, treated with and without urea after the antigen-antibody reaction. It is suggested that ELISA kit producers determine the suitable OD range for avidity determination and supply the user with this information.

Past TBEV infections showed regularly high avidity IgG, and most of the sera from current infections contained low avidity IgG during the first few weeks after the onset of clinical symptoms. Maturation of avidity showed a high degree of variability between individual sera; but in general, avidity maturation was much slower than the maturation of IgG directed against VCA of EBV. The slower maturation represents a practical advantage for routine serology, since sera taken within the first few weeks can still be recognized as indicating of current infection. Thus, low avidity indices may be taken as a clear indication of current TBEV infection during recent weeks, whereas high avidity IgG does not support this diagnosis. Therefore, low avidity IgG will reinforce the conclusion based on positive anti-TBEV IgG and IgM. High avidity IgG will contradict the conclusion based on the finding of positive IgM and point to a possible persistent IgM response. Persistent IgM has been reported to be a frequent problem in classical TBEV serology [Roggendorff et al., 1981; Hofmann et al., 1983a] and in other serological systems [Bauer, 1996]. In the absence of available presera, this situation cannot be recognized properly by conventional serology. The major advantage of avidity determination, however, lies in the detection of

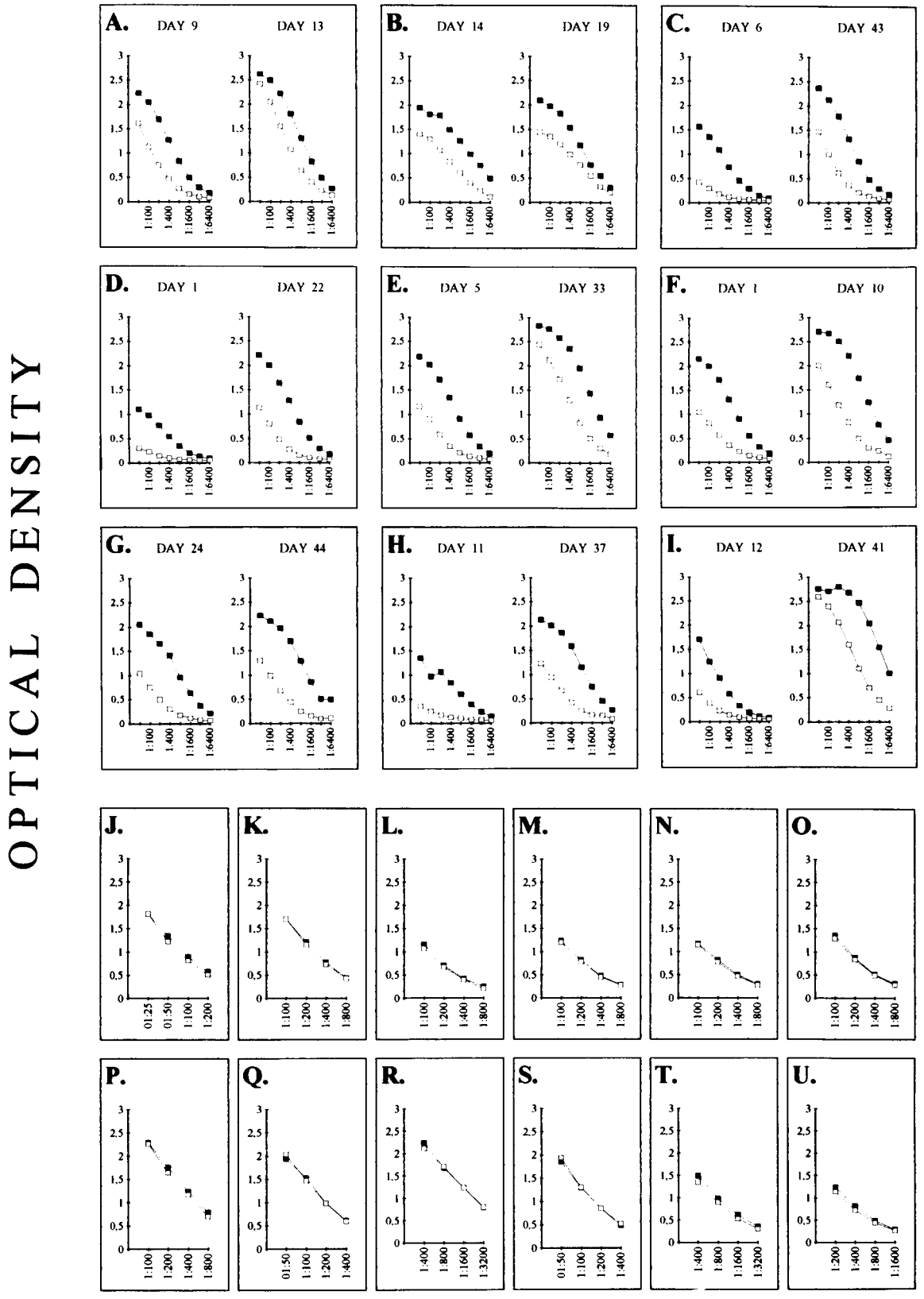


Fig. 3. Kinetics of maturation of IgG after primary TBEV infection. Eighteen sera from nine patients with current TBEV infection and defined time span with respect to the beginning of clinical symptoms as well as 12 sera from patients with past TBEV infection were serially diluted and incubated in a commercial TBEV ELISA under standard conditions. After the first incubation, assays were either processed as usual (closed symbols) or treated with 7 M urea in PBS for 3 min, washed three times, and then processed as usual. The OD values are plotted against the respective dilutions.

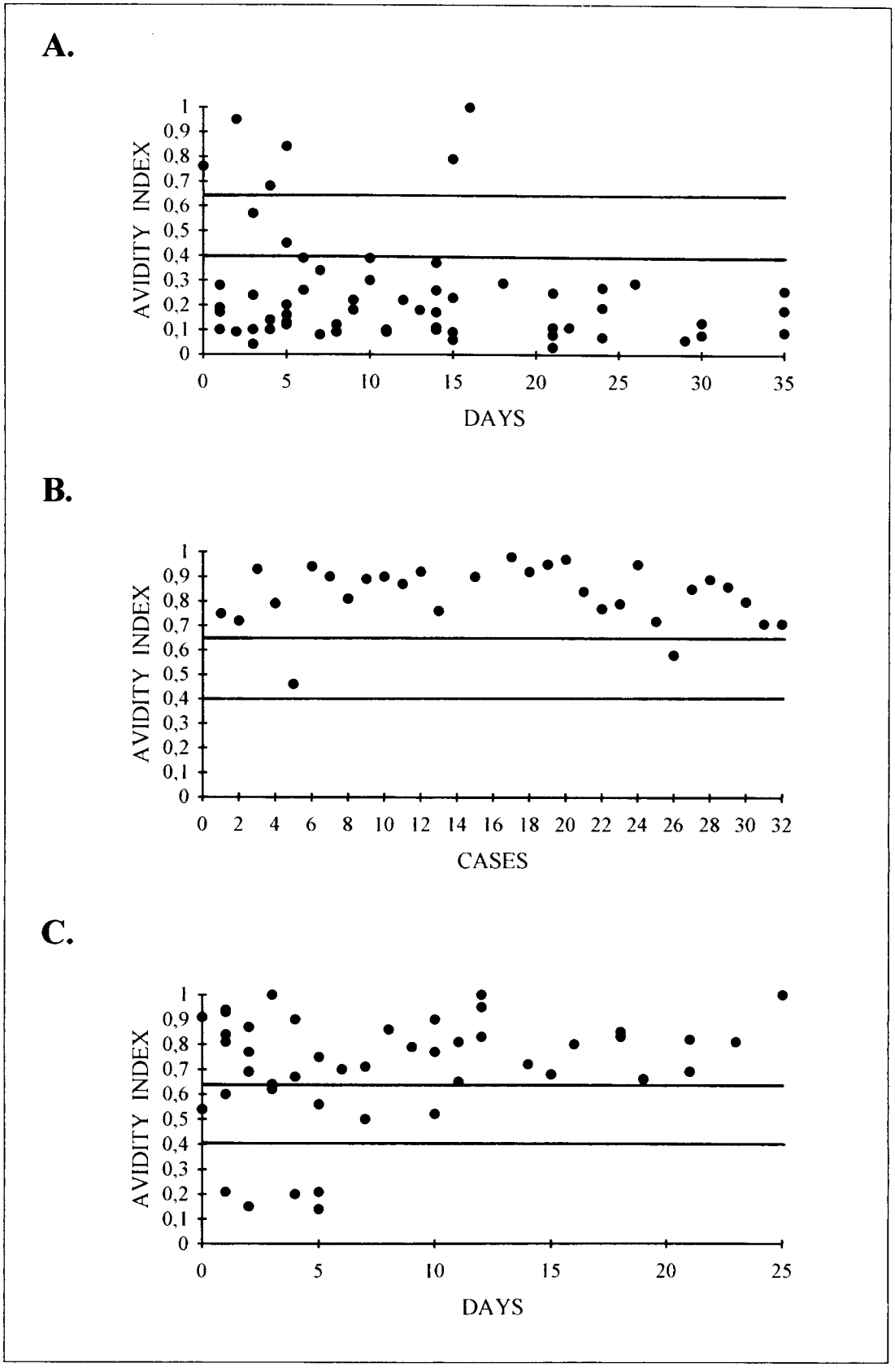
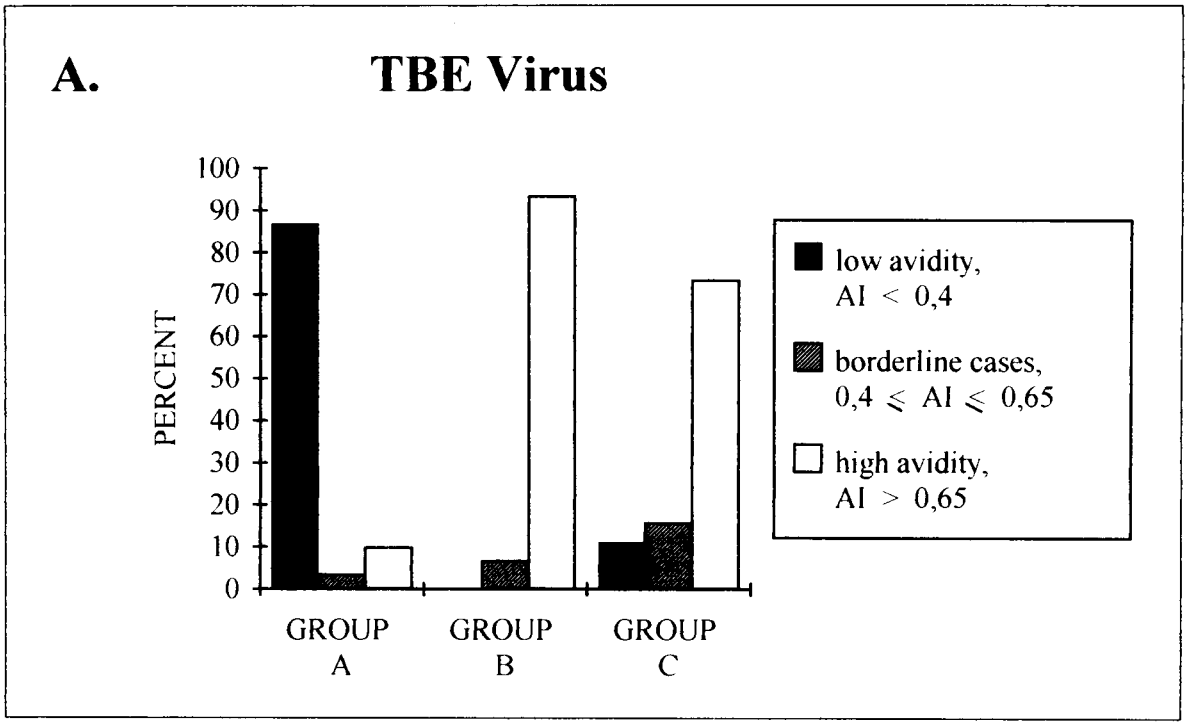


Fig. 4. Avidity determination in patients with current TBEV infection indicated by IgM, past infection and patients with clinical symptoms but without detectable IgM. Avidity indices of TBEV IgG of 61 cases with clinical symptoms of TBEV infection, positive TBEV IgG and IgM (**A**); 30 cases with past TBEV infection (**B**) and 45 cases with clinical symptoms of current TBEV infection but negative TBEV IgM in the presence of TBEV IgG (**C**) were tested for avidity of TBEV IgG. An avidity index of > 0.4 was defined as low and indicative or current infection; avidity indices between 0.4 and 0.65 were defined as being borderline, indices > 0.65 were defined as high and thus indicative of past infection. The groups were characterized by their subset of cases with low, borderline, and high avidity.



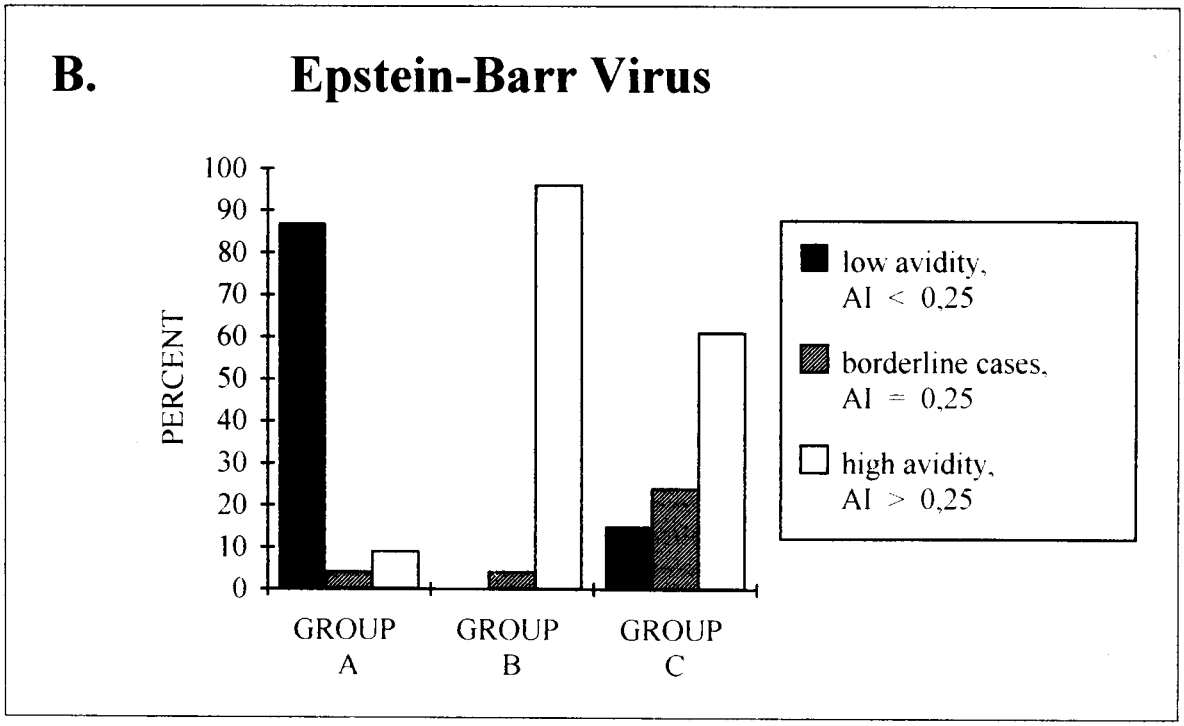


Fig. 5. Diagnostic significance of avidity determination for TBEV and EBV serology. The percentages of high, borderline, and low avidity cases for the three groups studied in Figure 4 were compared to the avidity indices determined for 96 cases of VCA-IgM-positive cases of primary EBV infection (**A**); 200 cases of past EBV infection (**B**) and 136 cases with the suspicion of primary EBV infection, negative anti-EBNA-1, but negative VCA-IgM [taken from Vetter et al., 1994].

current cases of TBEV without measurable IgM in the available serum samples. Our retrospective study of sera from cases with clinical symptoms of current TBEV infection but negative TBEV IgM showed, that five out of 45 cases could be recognized as current TBEV infections by avidity determination. After this study had been finished, two severe cases of TBEV infections diagnosed clinically without detectable anti-TBEV IgM, one of them with fatal outcome, were retrospectively diagnosed as current TBEV infections. This finding demonstrates the significance and the applicability of our method for routine TBEV serology.

It must be assumed that cases of current TBEV infection without detectable IgM sera certainly developed IgM during the interaction of their humoral immune system with virus infection. But there may be several reasons for a negative IgM result in the available sera: the IgM response may have been very low and the sera were not taken when IgM was at the maximum level. In the case of a low level and short serum IgM response, sera may have been taken too late. In the case of a delayed IgM response, sera may have even been taken too early. As avidity maturation is a unidirectional process, avidity determination is less prone to be hampered by similar problems and thus can result in more reliable results than classical IgM determination. The relatively high number of cases with borderline avidity for TBEV in the group of questionable cases (group C) indicates that they may represent cases where the sera had been taken relatively late after actual onset of clinical symptoms. Therefore, it seems that there remains a significant number of cases of recent TBEV infections that cannot be recognized, as the sera are already negative for IgM and exhibit borderline or high avidity. This problem can only be solved if physicians collect serum samples at the earliest possible time point. In certain cases, sera of borderline avidity of TBEV IgG may still help to diagnose recent TBEV infection, if control sera taken shortly later will allow to recognize continuous maturation of IgG avidity significantly above the borderline level. This approach will depend on very exact measurements and parallel tests of the sera to be compared.

Both in TBEV serology and EBV serology, avidity determination helped to recognize current or recent infections which were not identified by conventional serology, since the IgM response was already below the level of detectability. In both viral infections, there was a similar percentage of current cases of infection that were only recognizable by this particular method. This result points to the specific potential of avidity determination. It may be assumed that the situation is also similar in other viral infections. As an additional benefit, avidity determination may help to clarify cases of persistent IgM response, reactivated IgM or IgM due to polyclonal stimulation. These IgM responses might otherwise readily be mistaken as indications for current infection. Therefore, avidity determination should be considered as an additional requirement or routine serology. If avidity determination will be applied adequately, the extra costs will be balanced by relatively clear diagnostic conclusions that prevent unnecessary further serological testing.

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